

# Elastin Stabilization for Treatment of Abdominal Aortic Aneurysms

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**Background**—Maintaining the integrity of arterial elastin is vital for the prevention of abdominal aortic aneurysm (AAA) development. We hypothesized that in vivo stabilization of aortic elastin with pentagalloyl glucose (PGG), an elastin-binding polyphenol, would interfere with AAA development.

**Methods and Results**—Safety and efficacy of PGG treatment were first tested in vitro using cytotoxicity, elastin stability, and PGG-elastin interaction assays. For in vivo studies, the efficacy of PGG was evaluated within a well-established AAA model in rats on the basis of CaCl<sub>2</sub>-mediated aortic injury. With this model, PGG was delivered periadventitially at 2 separate time points during the course of AAA development; aortic diameter, elastin integrity, and other pathological aspects were monitored and evaluated in PGG-treated aortas compared with saline-treated control aortas. Our results show that a one-time periadventitial delivery of noncytotoxic levels of PGG inhibits elastin degeneration, attenuates aneurysmal expansion, and hinders AAA development in rats without interfering with the pathogenic mechanisms typical of this model, namely inflammation, calcification, and high metalloproteinase activities. PGG binds specifically to arterial elastin and, in doing so, preserves the integrity of elastic lamellae despite the presence of high levels of proteinases derived from inflammatory cells.

**Conclusions**—Periadventitial administration of PGG hinders the development of AAA in a clinically relevant animal model. Stabilization of aortic elastin in aneurysm-prone arterial segments offers great potential toward the development of safe and effective therapies for AAAs. (*Circulation*. 2007;115:1729-1737.)

**Key Words:** aneurysm ■ aorta ■ drug delivery systems ■ elasticity ■ metalloproteinases ■ prevention ■ tannins

Abdominal aortic aneurysms (AAAs) are associated with impaired arterial wall integrity, leading to abnormal ballooning and eventual fatal rupture. AAAs, which are apparently increasing in frequency, have been cited as one of the top 10 causes of death among older men.<sup>1</sup> After initial diagnosis, AAA patients (AAA diameter  $\geq 2.5$  cm) are monitored for an increase in aortic diameter, and surgery aimed at preventing death from enlarged or ruptured AAAs is recommended when the diameter reaches  $\geq 5.5$  cm.<sup>2</sup> No pharmacological treatment currently exists for AAAs. Surgical procedures entail either endovascular stent graft repair or complete replacement of the diseased aortic segment with an artificial vascular graft. Although often effective, endovascular stents are anatomically appropriate for only 30% to 60% of AAA patients at the outset and present the risk of endoleaks and graft displacement.<sup>3</sup> Moreover, open surgery for full-size graft insertion is highly invasive, limiting its use to those patients with high operative risk.

## Clinical Perspective p 1737

Treatment options are particularly limited for patients with small or moderate aneurysms; this group makes up the largest percentage of all AAA patients.<sup>4</sup> This number is likely to increase dramatically with the advent of “blanket” screening of asymptomatic subjects with imaging surveillance.<sup>5</sup> Consequently, novel therapeutic approaches targeted at hindering the progression of AAAs promptly after diagnosis would be extremely beneficial.

Information about AAA mechanisms is currently gathered from analysis of late-stage aneurysmal samples obtained from patients and animal models. In addition to arterial dilatation, AAAs are characterized by degeneration of the arterial architecture,<sup>6</sup> decreased medial elastin content,<sup>7</sup> disruption or fragmentation of elastic lamellae,<sup>8</sup> presence of matrix-degrading enzymes such as matrix metalloproteinases (MMPs),<sup>9,10</sup> inflammatory infiltration,<sup>10</sup> and often calcification.<sup>11</sup> As a result of its multifactorial pathogenesis, antiin-

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flammatory agents,<sup>12</sup> proteinase inhibitors (such as tissue inhibitors of metalloproteinases [TIMPs]), and genetic and pharmacological inhibition of MMPs<sup>13</sup> have been tested as potential AAA treatments in experimental animals, but none has yet reached clinical application. Because long-term, adequate control of local inflammation and MMP activities may be difficult to achieve and may be accompanied by adverse side effects,<sup>14</sup> our hypothesis was that stabilization of aortic elastin in aneurysm-prone arterial segments offers potential for the development of safe and effective therapies for AAAs.

In current studies, we have explored polyphenolic tannins, specifically pentagalloyl glucose (PGG), as novel elastin-stabilizing agents. Tannins bind to elastin<sup>15</sup> and, in doing so, render it resistant to enzymatic degradation.<sup>15–17</sup> We provide evidence that periarterial treatment with PGG preserves elastin fiber integrity and hinders aneurysmal dilatation of the abdominal aorta in a clinically relevant animal model of AAA.

## Methods

Please see the online Data Supplement for expanded Methods.

### Pentagalloyl Glucose

High-purity PGG used throughout the *in vitro* and *in vivo* experiments was produced from tannic acid by methanolysis, as described previously.<sup>17</sup>

### In Vitro Cytotoxicity

Primary rat aortic smooth muscle cells and rat skin fibroblasts were exposed to increasing concentrations of PGG and viability assessed by the soluble tetrazolium salt (MTS) assay (Promega, Madison, Wis) and Live-Dead staining (Molecular Probes, Eugene, Ore). Cells also were incubated with PBS and 70% ethanol as negative and positive controls, respectively.

### In Vitro Efficacy

Native abdominal aortas collected from adult rats were treated *in vitro* with increasing concentrations of PGG dissolved in saline. Control samples were treated with saline alone. Aortic samples were tested for resistance to elastase by desmosine analysis.<sup>15,18</sup> To demonstrate PGG–aorta interactions, samples were stained with a phenol-specific histology stain and were tested for natural recoil ability using opening-angle measurements, as described previously.<sup>17,19</sup>

### Animal Surgeries

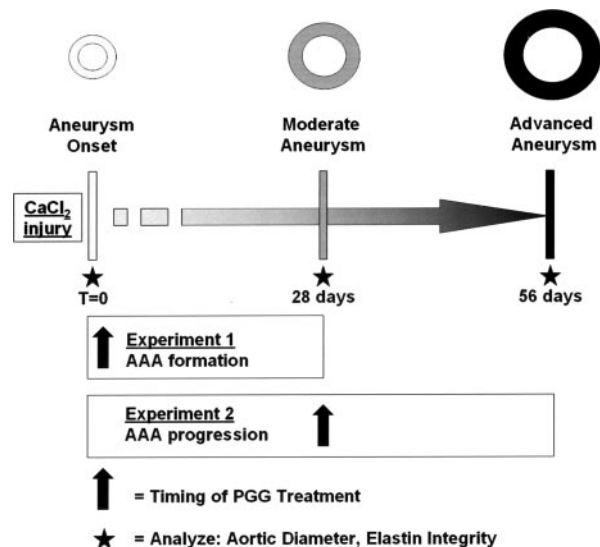
Two experiments were designed to evaluate the *in vivo* efficacy of PGG in hindering AAA formation and progression in a CaCl<sub>2</sub> injury model of aortic aneurysm (Figure 1).

#### Experiment 1: Interference With Formation of Early AAA

Infrarenal abdominal aortas of adult male Sprague-Dawley rats were perivascularly treated for 15 minutes with PGG dissolved in physiological saline using a presoaked gauze applicator. After rinsing, the aortas underwent chemical injury by application of 0.5 mol/L CaCl<sub>2</sub> for 15 minutes. Control aortas were treated with vehicle (saline) for 15 minutes, rinsed, and then subjected to CaCl<sub>2</sub>. After 28 days, rats were humanely euthanized with CO<sub>2</sub> asphyxiation, and aortas were excised and analyzed as described below. Changes in external aortic diameter were measured by digital photography.

#### Experiment 2: Hindrance of AAA Progression

Abdominal aortas were treated perivascularly 0.5 mol/L CaCl<sub>2</sub> to induce AAA, and rats were allowed to recover. After 28 days, the aneurysmal aortas were reexposed and treated with PGG in saline for 15 minutes, as described above. In the control group, aneurysmal aortas were treated with vehicle (saline) for 15 minutes. At 28 days after the second surgery (56 days after initial CaCl<sub>2</sub> injury), rats were humanely euthanized, and samples were collected for analysis.



**Figure 1.** Experimental design for *in vivo* experiments. In experiment 1, PGG was applied to healthy abdominal aorta immediately before CaCl<sub>2</sub>-mediated injury, and AAA development was followed for 28 days. In experiment 2, PGG was applied to aneurysmal aorta, and development of advanced AAA was monitored for another 28 days.

Changes in external aortic diameter were measured by digital photography.

All animals used in our experiments received humane care in compliance with protocols approved by the Clemson University Animal Research Committee as formulated by the National Institutes of Health (publication No. 86-23, revised 1999).

### Elastin Content and Integrity Assessment

Aortic segments were analyzed for desmosine content by radioimmunoassay<sup>18</sup> and by histology using Verhoeff van Gieson stain for elastin and hematoxylin and eosin for general structure.

### PGG Binding Studies

To extract PGG, aortas retrieved 28 days after PGG treatment (experiment 1) were pulverized and shaken with 80% methanol; extracts were assayed for phenol content with the Folin-Denis reaction.<sup>20</sup> As positive controls, rat aortas were treated with PGG *in situ* for 15 minutes and extracted for analysis of initial PGG content.

### Calcification Assessment

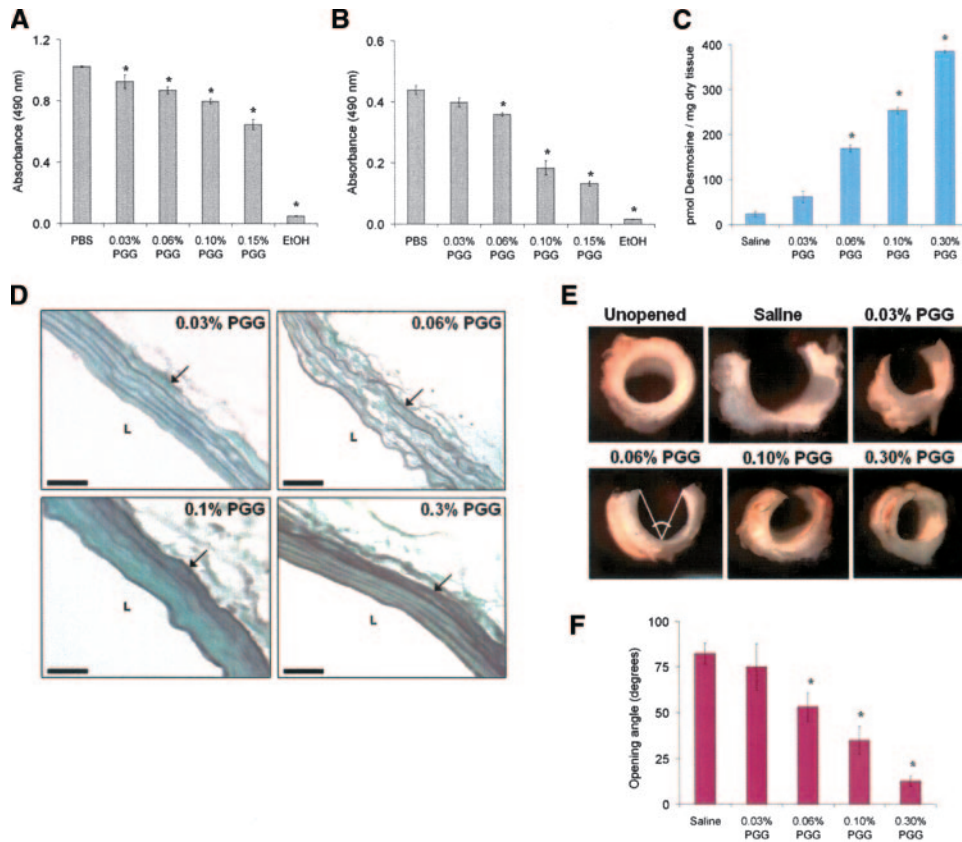
Rat aortic samples retrieved at 28 days after CaCl<sub>2</sub> injury (experiment 1) were analyzed for calcium content using atomic absorption spectrophotometry and by histological Alizarin red staining, as described before.<sup>21</sup>

### MMP and TIMP Quantification

MMP-2 and MMP-9 activities in rat aortas retrieved 28 days after CaCl<sub>2</sub> injury (experiment 1) were analyzed by gelatin zymography.<sup>22</sup> TIMP-2 levels were assayed by ELISA (Amersham Biosciences, Piscataway, NJ).

### Immunohistochemistry

Macrophages were detected on samples from control (saline) and PGG-treated aortas 28 days after treatment (experiment 1) using a mouse anti-rat monocyte/macrophage primary antibody (Chemicon International, Temecula, Calif), whereas lymphocytes were detected with a rabbit anti-CD3 T-cell primary antibody (Sigma-Aldrich, St Louis, Mo). Staining was performed with the appropriate biotinylated secondary antibodies and an avidin-biotin detection system (Vector Laboratories, Burlingame, Calif).



**Figure 2.** In vitro safety and efficacy studies of PGG interactions with native rat abdominal aorta. Rat aortic smooth muscle cells (A) and rat skin fibroblasts (B) were exposed to increasing concentrations of PGG, and cytotoxicity was measured with the MTS assay ( $n=3$  per group per cell type). Cells exposed to PBS and 70% ethanol (EtOH) were used as negative and positive controls, respectively. C, To test resistance to elastase, aortic samples were exposed to increasing concentrations of PGG for 15 minutes in vitro and then exposed to pure elastase. Aortic elastin content was measured in each group by desmosine analysis ( $n=5$  per group). D, For histological confirmation of PGG binding to aortic elastin, PGG-treated aortic tissues were stained with a phenol-specific stain (black indicates PGG) and counterstained with light green (L indicates lumen; bar=50  $\mu\text{m}$ ). Arrows point to elastic lamellae. E, To test elastic recoil, aortic rings were treated for 15 minutes with increasing concentrations of PGG and subjected to ring-opening analysis ( $n=5$  per group). Opening angles were measured graphically (bottom left corner). F, Mean opening angles for each group are shown as a function of PGG concentration. \*Statistically significant differences ( $P<0.05$ ) relative to the negative control group.

### Statistical Analyses

Results are expressed as mean  $\pm$  SEM. Statistical analyses of the data were performed using single-factor ANOVA. Subsequently, differences between means were determined through the use of the least significant difference with an  $\alpha$  value of 0.05.

The authors had full access to and take responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

## Results

### In Vitro Safety and Efficacy Studies

In preparation for the animal studies, several experiments were performed in vitro to evaluate the effects of a single 15-minute application of PGG on cells and arterial extracellular matrix, specifically elastin. MTS results (Figure 2A and 2B) showed that exposure of cells to PGG concentrations of up to 0.06% had minimal cytotoxic effects. No statistical differences were observed between cells exposed to 0.03% and 0.06% PGG ( $P>0.05$ ). These results were also confirmed by Live-Dead assay (data not shown). Desmosine analysis of aortic samples exposed to elastase showed an increasing trend in elastin preservation with increasing PGG concentrations (Figure 2C). Moreover, the intensity of polyphenol histolog-

ical staining increased progressively with increasing concentrations of PGG, revealing direct binding of PGG to elastic fibers (Figure 2D).

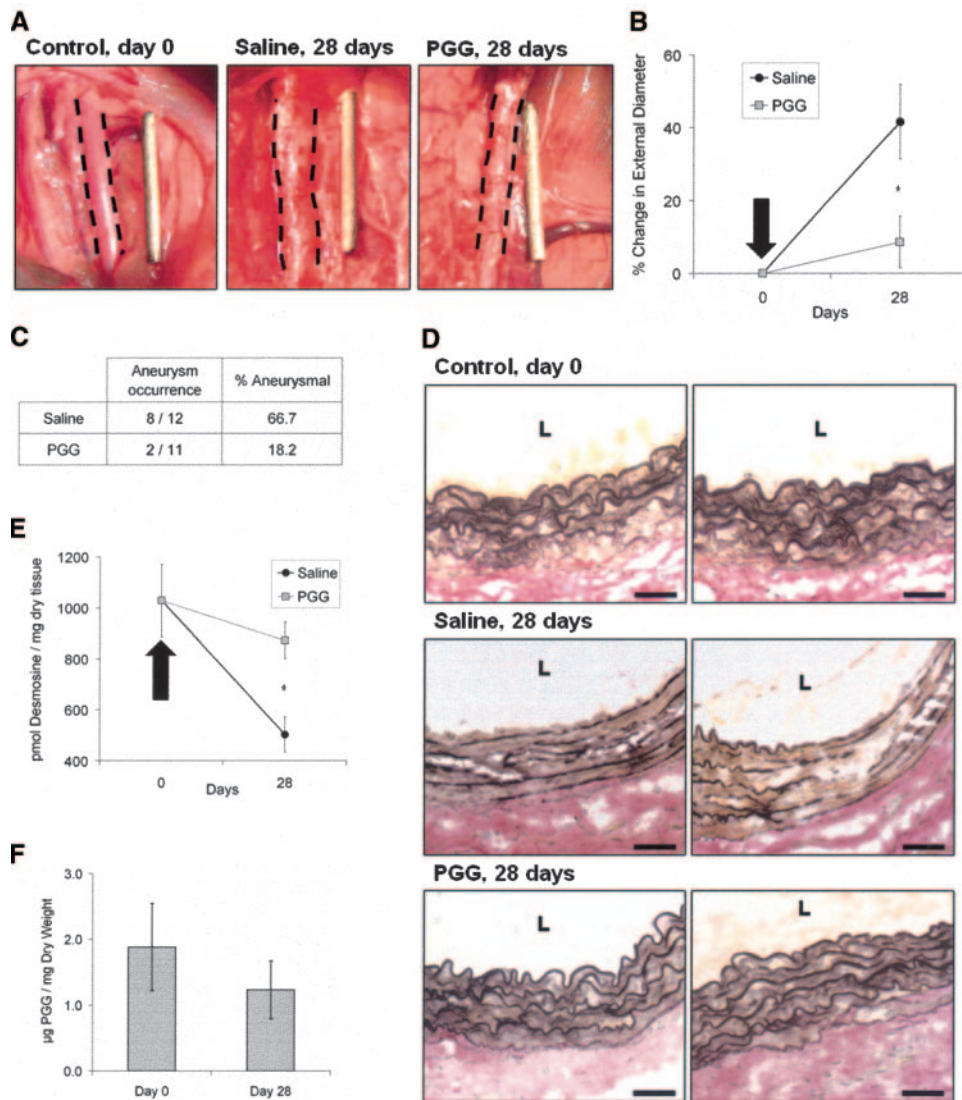
For further proof of PGG-arterial elastic fiber interactions, the ring-opening test was performed on PGG-treated native rat aortic rings (Figure 2E and 2F). When allowed to open by a single incision, rings extended to  $>75^\circ$ , revealing the natural elastic recoil properties of aortic tissues. Rings that were exposed to increasing concentrations of PGG exhibited progressively smaller opening angles, suggestive of the direct interactions of PGG with elastic fibers.

Having established that a 15-minute exposure to PGG solutions of up to 0.06% is not cytotoxic and effectively stabilizes aortic tissue by binding to elastic fibers, we designed 2 experiments to test the in vivo efficacy of PGG in hindering AAA formation and progression (Figure 1).

### Perivascular Delivery of Noncytotoxic Levels of PGG Hinders Aneurysm Formation

In experiment 1, perivascular application of  $\text{CaCl}_2$  to the infrarenal abdominal aorta induced significant changes in aortic diameter at 28 days after injury (Figure 3A and 3B).

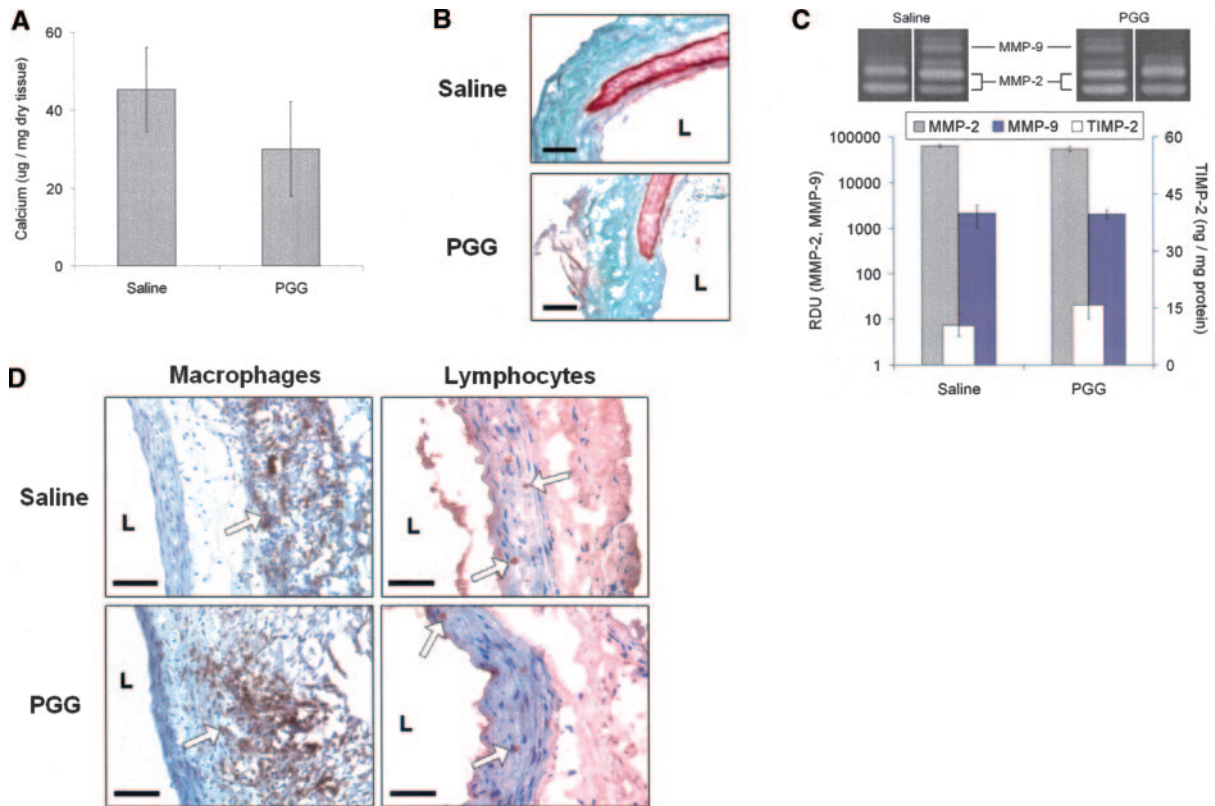




**Figure 3.** Local delivery of PGG prevents AAA development and elastin degeneration. Adult rat abdominal aortas were treated periadventitially with PGG for 15 minutes, rinsed, and then exposed to  $\text{CaCl}_2$ -mediated chemical injury to induce aneurysm formation. In control rats, PGG treatment was replaced with saline, followed by  $\text{CaCl}_2$ . A, External diameter of the infrarenal aorta between the renal artery (top) and iliac bifurcation (bottom) was measured by digital photography. Black dashed lines were added to aid in the identification of aortic anatomy. B, Mean percent change in diameter at 28 days relative to day 0 ( $n=12$  for saline controls,  $n=11$  for PGG). C, Calculated aneurysm occurrence using an arbitrary 20% diameter increase threshold for defining AAA. D, Elastin integrity evaluated by histology using VVG stain (black L indicates lumen; elastin stained black; bar = 50  $\mu\text{m}$ ) and (E) desmosine analysis. F, Quantitative PGG content analysis in explanted aorta ( $n=3$ ). Thick black arrows in B and E designate timing of PGG application. \*Statistically significant differences between means ( $P<0.05$ ) at 28 days relative to saline-treated controls.

Comparative measurements of control rats at days 0 and 28 after surgery ( $1.3 \pm 0.05$  and  $1.9 \pm 0.1$  mm, respectively) revealed a mean increase in diameter of  $42 \pm 10\%$  ( $P<0.05$ ;  $n=12$ ). In comparison, aortas exposed to PGG exhibited a minimal ( $8 \pm 7\%$ ) increase in diameter after 28 days (from  $1.5 \pm 0.06$  to  $1.6 \pm 0.09$  mm). With an arbitrary threshold of a 20% diameter increase considered aneurysmal in this experimental model, 8 of 12 rats exhibited aneurysms in the control group (66.7%), and only 2 of 11 rats were aneurysmal in the PGG group (18.2%; Figure 3C). These results indicate that PGG application to healthy aorta effectively hindered formation of aneurysms in this experimental model.

As shown above, exposure to PGG had minimal in vitro cytotoxic effects on rat cells. In our animal studies, PGG-treated rats did not exhibit significant weight losses during the 28-day test period ( $42.3 \pm 3.4$  g gain in the control group versus  $44.2 \pm 3.6$  g in the PGG group;  $P<0.05$ ;  $n=12$ ). Liver samples did not exhibit any noticeable histological changes indicative of hepatotoxicity (data not shown). In addition, levels of serum ALT (an enzyme used to assess liver function) for saline controls ( $9.1 \pm 1.5$  U/L) and PGG-treated rats ( $17.7 \pm 3.1$  U/L) were consistently within the acceptable range (5 to 45 U/L). Furthermore, ALT levels for PGG-treated rats were not statistically different from those of nonsurgery controls rats ( $11.5 \pm 1.2$  U/L;  $P>0.05$ ), suggesting



**Figure 4.** PGG application does not interfere with pathogenic mechanisms of AAA formation. A, Aortic calcification at 28 days after CaCl<sub>2</sub> injury was evaluated by calcium content analysis (n=12 per group) and (B) histology using Alizarin red stain (red L indicates lumen; calcium deposits stained red). C, MMP-2 and MMP-9 enzymatic activities and TIMP-2 levels in the 2 groups were not statistically different (*P*>0.05; n=6 per group) at 28 days after injury. D, Immunohistochemistry for macrophages (bar=100 μm) and lymphocytes (bar=50 μm). For both types, positive reaction is brown.

that PGG treatment did not induce major liver damage within this model.

**Treatment With PGG Prevents Degeneration of Aortic Elastin**

In parallel with aortic dilatation, experimentally induced AAAs were accompanied by major changes in vascular elastin content and integrity as shown by desmosine analysis and histology (Figure 3D and 3E). Compared with nonsurgery control aorta, aortic elastin content in the control group diminished by almost 50% (Figure 3E) and exhibited characteristic flattening and fragmentation of the elastic laminae (Figure 3D) at 28 days after injury.

Conversely, aortas from the PGG group exhibited minimal (<15%) decrease in elastin content (Figure 3E) and excellent preservation of elastic lamina integrity and waviness (Figure 3D), suggesting that PGG delivery effectively prevented elastin degeneration in this animal model.

**PGG Binds to Aortic Elastin In Vivo**

The affinity of phenolic tannins toward elastin is well known from histology techniques<sup>23</sup> and was previously demonstrated in vitro with pure elastin.<sup>15</sup> Compared with day 0 (1.8±0.6 μg PGG/mg dry tissue), aortas explanted 28 days after PGG application contained slightly lower (Figure 3F) but not statistically different amounts of PGG (1.2±0.4 μg PGG/mg dry tissue; *P*>0.05), indicating that in vivo binding of PGG to

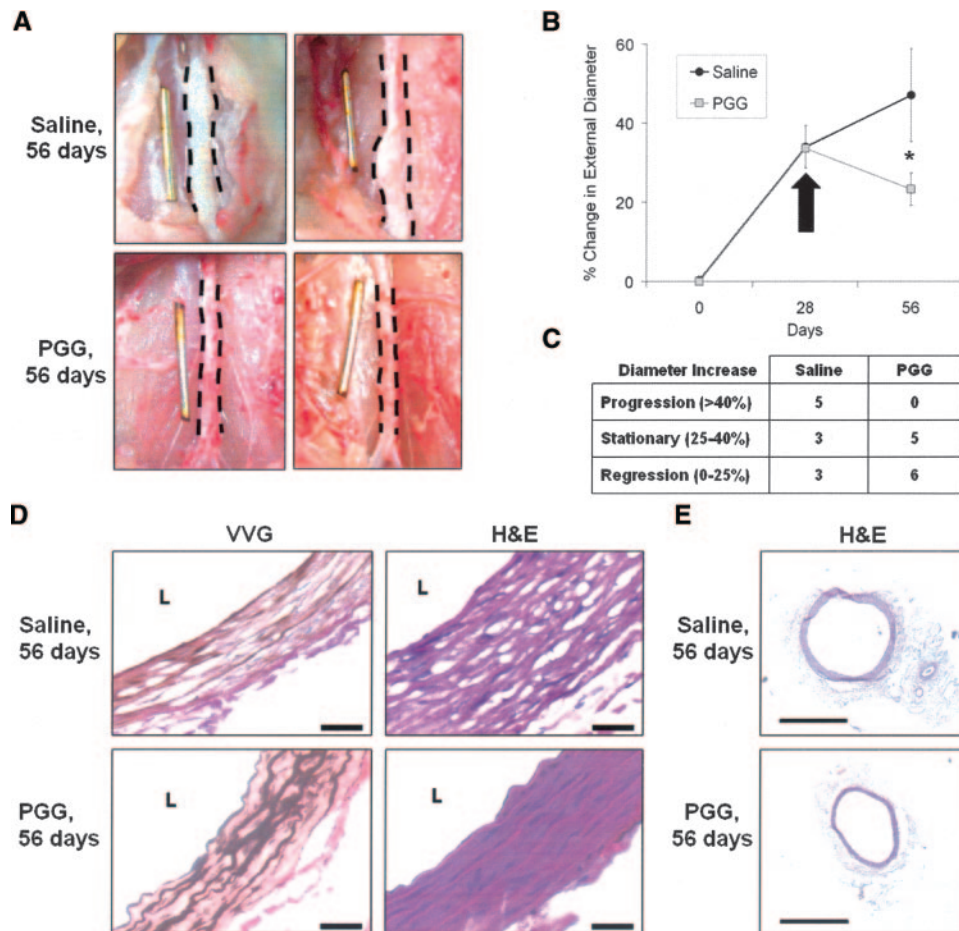
aortic tissues is stable for a minimum of 28 days in this animal model.

**Treatment With PGG Does Not Interfere With Model-Related Pathogenesis**

To investigate pathogenic aspects typical to this animal model,<sup>24</sup> calcium content, macrophage infiltration, MMP activities, and expression of TIMP were analyzed in aortas retrieved 28 days after CaCl<sub>2</sub> treatment (experiment 1). Perivascular injury induced significant tissue calcification after 28 days (Figure 4A), which was localized mainly in the media (Figure 4B). Similar calcium values (*P*>0.05) and distribution were observed in PGG-treated aortas. MMP activities and TIMP-2 levels (Figure 4C) also were not different between controls and PGG-treated aortas (*P*>0.05), suggesting that tissues in both groups were exposed to similar proteolytic environments. Moreover, hematoxylin and eosin and immunohistochemical staining for macrophages and lymphocytes revealed comparable inflammatory infiltrates in both groups (Figure 4D). Taken together, these results suggest that PGG treatment did not interfere with key pathogenic mechanisms typical of this AAA experimental model.

**Periadventitial Treatment of Aneurysmal Aorta With PGG Limits AAA Progression**

In experiment 2, rat aortas were treated with CaCl<sub>2</sub>, and AAA was allowed to develop for 28 days. At this time point, the



**Figure 5.** Delivery of PGG to aneurysmal aorta prevents AAA progression. Adult rat abdominal aortas were treated periadventitially with  $\text{CaCl}_2$ , and AAAs were allowed to develop for 28 days. At that time, aneurysmal aortas were reexposed and treated with PGG. In control rats, PGG treatment was replaced with saline. Rats in both groups were euthanized 28 days after the second surgery (total, 56 days after initial  $\text{CaCl}_2$  injury). **A**, External diameter of the infrarenal aorta was measured by digital photography after perfusion fixation. Black dashed lines were added to aid in the identification of aortic anatomy. **B**, Mean percent change in diameter at 28 and 56 days relative to day 0 ( $n=11$  per group). Thick black arrow designates timing of PGG application. **C**, Numbers of individual rats that exhibited increased, stationary, or reduced aortic diameters at 56 vs 28 days. **D**, Elastin integrity and tissue architecture were evaluated by histology using VVG stain (black L indicates lumen; elastin stained black) and hematoxylin and eosin. Bar= $40\ \mu\text{m}$ . **E**, Low-magnification pictures used to digitally measure the thickness of arterial media. Bar= $1000\ \mu\text{m}$ . \*Statistically significant differences between means ( $P<0.05$ ).

aneurysmal aortas were exposed through a second surgery, and PGG was applied perivascularly (PGG group). Saline was applied to aneurysmal aortas in the control group. AAA progression was followed for another 28 days in both groups. A progressive diameter increase, reaching a mean  $47.1\pm 11\%$  increase at 56 days, was measured in the control group (Figure 5A and 5B). Approximately half of the aneurysmal aortas significantly increased in diameter from day 28 to 56, indicating chronic AAA progression in this animal model (Figure 5C).

Conversely, aneurysmal aortas that were exposed to PGG exhibited no increase in mean diameter at 56 days compared with day 28 mean values (Figure 5B). It is especially noteworthy that 100% of aortas in the PGG group (11 of 11) maintained the same diameter or exhibited a decrease in aortic diameter at 56 compared with 28 days (Figure 5C). The mean diameter value at 56 days for the PGG group was actually slightly lower than that at 28 days but not statistically

significant ( $P>0.05$ ). Overall, these results indicate that PGG application to aneurysmal aortas effectively hindered arterial dilatation in this experimental model.

#### Effect of PGG on Degeneration of Aortic Elastin

At 56 days after injury, aneurysmal aortas exhibited extensive flattening, fragmentation, and degeneration of the elastic laminae in the control group (Figure 5D). Overall tissue architecture was indicative of severe tissue degeneration as outlined by numerous gaps or lacunae, bestowing the aneurysmal aorta with a porous, "spongy" aspect. In contrast, PGG-treated aortas exhibited improved preservation of elastic lamina integrity and waviness and overall preserved tissue architecture (Figure 5D). Analysis of vascular elastin revealed no significant differences in elastin content as shown by desmosine analysis ( $679.5\pm 63.2$  and  $715.9\pm 90.6$  pmol desmosine/mg dry tissue). Moreover, these elastin values were not statistically different from the 28-day values



(503.0±69.2 pmol desmosine/mg dry tissue;  $P>0.05$ ), indicating that the severe elastin degradation observed at 28 days has reached a steady state and has not changed significantly as AAA progressed from 28 to 56 days. Morphometric measurements on perfusion-fixed aortas (Figure 5E) revealed that the thickness of the aortic media was significantly smaller in the PGG treatment group (55.4±2.4  $\mu\text{m}$ ) compared with saline-treated aneurysmal controls (80.3±4.7  $\mu\text{m}$ ;  $P<0.05$ ). Taken together, these results suggest that PGG treatment of aneurysmal aortas effectively prevented chronic diameter expansion and aortic matrix deterioration in this AAA animal model.

### Discussion

Aneurysms arise from irreversible, chronic pathological changes that involve degeneration of structural matrix components. The role of MMP/TIMP imbalances in AAA initiation and development has been irrevocably demonstrated by studies using MMP- and TIMP-knockout mice.<sup>25–27</sup> MMP-mediated loss of elastin, a hallmark of AAA, is believed to lead to progressive dilatation of the aorta. Therefore, our studies focused on chemical stabilization of vascular elastin as a novel approach to limit aneurysmal degeneration.

The traditional approach to treating AAAs involves surgical procedures such as endovascular stent graft repairs or complete replacement of the diseased section of the aorta. Nonsurgical approaches, currently still in the experimental phase, include use of antiinflammatory agents, proteinase inhibitors, and genetic and pharmacological inhibition of MMPs in animal models. Doxycycline, an antibiotic and MMP inhibitor, showed initial promising results in a pilot clinical study.<sup>28</sup> Recently, Yoshimura et al<sup>29</sup> reported that the systemic pharmacological inhibition of c-Jun N-terminal kinase, an intracellular signaling switch that controls MMP production, might block AAA progression and stimulate aneurysm regression.

Although numerous attempts have focused on proteolytic inhibition for AAA treatment, we present a radically innovative approach, namely periaortic delivery of nontoxic concentrations of phenolic tannins such as PGG to stabilize elastin and render it resistant to enzymatic degradation. In previous studies, we have shown that tannic acid (a decagalloyl glucose) binds to pure aortic elastin and porcine aortic wall, in both cases resulting in improved resistance to enzyme-mediated elastin degradation.<sup>15,16</sup> More recently, we have reported that PGG, a more stable and less cytotoxic derivative of tannic acid, also binds to and protects elastin within porcine aortic wall with very similar efficacy.<sup>17</sup> Our hypothesis was that the ability of PGG to stabilize elastin would extend in vivo to rat aorta. To verify our hypothesis, we performed a series of in vitro safety and efficacy studies, followed by in vivo validation in a rat AAA model.

### Safety and Efficacy Studies

Results showed that PGG binds specifically to vascular elastin and, in doing so, renders elastin resistant to enzymatic degradation. In addition to being an effective elastin-stabilizing agent, PGG, when used at concentrations of up to

0.06%, had virtually no adverse effects on cell viability when applied in vitro to rat cells.

### Local PGG Delivery Hinders AAA Formation and Limits Diameter Increase

Traditionally, an increase in aortic diameter is considered the defining characteristic of AAA. Numerous investigators studied AAA formation in the  $\text{CaCl}_2$  injury model in mice,<sup>30</sup> rabbits,<sup>11,31</sup> and rats.<sup>32</sup> We have previously used this model to show that  $\text{CaCl}_2$  injury induces a series of pathogenic alterations that share many similarities to human pathology and to changes observed in other experimental animals.<sup>24</sup> In experiment 1, application of  $\text{CaCl}_2$  to the rat infrarenal abdominal aorta induced a mean increase in diameter of 42% at 28 days after application. The extent of aneurysmal dilatation observed in our studies corresponds well with other published studies using  $\text{CaCl}_2$  injury that consistently reported a 25% to 75% increase in diameter in rodents.<sup>25,26,32,33</sup> In our studies, this pathology was observed in 8 of 12 control rats (66% incidence). This prevalence also was similar to other studies that reported AAA incidences of 60% to 80%.<sup>25,26</sup> It is unknown why a consistent number of genetically similar experimental animals are apparently resistant to  $\text{CaCl}_2$ -mediated injury. This aspect warrants further investigation.

Conversely, mean aortic diameter increase in the PGG group was <10%, which is traditionally not considered indicative of aneurysm formation. The overall AAA incidence in the PGG group was <20%, indicating that in this model, PGG prevented aneurysm formation in more than 8 of 10 animals. Although the detailed mechanisms of this successful approach are not entirely known, we provide evidence that it likely involves PGG-mediated elastin stabilization.

In addition to dilatation, AAAs also are characterized by progressive damage to arterial elastin. In our experimental model, periaortic application of  $\text{CaCl}_2$  induced a mean decrease of >50% in aortic elastin content. Earlier studies have shown that elastin degeneration starts within the first days after  $\text{CaCl}_2$  injury and that this chronic process is related to an overexpression of MMPs within the aortic extracellular matrix.<sup>24</sup> Similar to diameter increase, the incidence of elastin degeneration in  $\text{CaCl}_2$ -treated aorta was observed in >80% of control rats. Typically, aortic samples that were characterized by low elastin content also exhibited characteristic flattening and fragmentation of the naturally wavy elastic lamellae. This histological aspect is characteristic of the aortic pathology in  $\text{CaCl}_2$ -treated aorta.<sup>29</sup> It is not clear at this time what the mechanism and clinical significance of elastic fiber flattening are, and this aspect merits further examination.

Periaortic treatment of aorta with PGG resulted in remarkable preservation of elastin integrity. Quantitative desmosine results showed that elastin content in the PGG group was not statistically different from the nonsurgery controls ( $P<0.05$ ), indicating minor, if any, elastin degradation. This was accompanied by excellent histological preservation of elastic lamellar integrity and waviness. Elastin preservation in the  $\text{CaCl}_2$  experimental model also was observed in aortas of MMP-knockout mice<sup>25,26</sup> and in mice treated systemically with a c-Jun N-terminal kinase inhibitor.<sup>29</sup> In all studies published thus far, the present report

included, elastin preservation was consistently associated with the absence of aneurysm initiation or progression. This indicates that elastin stabilization by either MMP inhibition or PGG treatment may be effective as a potential treatment of AAAs.

### Periadventitial Treatment of Aneurysmal Aorta With PGG Limits AAA Progression

In a more clinically relevant experiment, PGG was directly applied to aneurysmal aortas, and AAA progression was followed after this surgical intervention. Although most control aneurysmal aortas (saline treated) continued to expand in diameter, all of the PGG-treated aneurysmal aortas maintained the same diameter or exhibited a decrease in aortic diameter at the end of the experiment (56 days). Diameter expansion in aneurysmal controls was associated with changes in medial thickness and integrity that included severe tissue degeneration. Yoshimura et al<sup>29</sup> reported similar inhibition of AAA progression after aneurysm onset in rodent animal models as a result of systemic pharmacological inhibition of c-Jun N-terminal kinase. Because c-Jun N-terminal kinase is an intracellular signaling switch that controls MMP production, its inhibition hindered MMP production and thus prevented degeneration of the vascular matrix.

In our studies, histological analysis of aneurysmal aortas at day 56 showed a significant decline in elastin integrity in the control group, indicating that elastin degeneration is a chronic pathogenic aspect characteristic of this AAA model. Hallmarks of this process were elastic fiber flattening, fragmentation, and lack of elastic lamina staining in extended areas in almost all samples (5 of 6). Aortas from the PGG group exhibited improved preservation of elastic lamina integrity and waviness in most samples (4 of 6). Desmosine analysis for this experiment revealed little quantitative difference for the PGG group compared with controls, possibly because elastin degradation has reached a maximum threshold at 28 days. Being mindful of these limitations, we nonetheless suggest that PGG-mediated elastin stabilization in aneurysmal aortas has the potential to significantly hinder AAA development.

### Mechanisms of PGG-Mediated Elastin Stabilization

As demonstrated before, PGG exhibits an outstanding affinity toward elastin, possibly binding to hydrophobic areas, which are known to be susceptible to protease-mediated elastolysis.<sup>16</sup> In the present studies, we have validated PGG binding to aortic elastin *in vivo* and provided evidence that the binding is stable for a minimum of 28 days in this accelerated AAA experimental model. We also have provided ample *in vitro* evidence that binding of PGG to arterial elastin provides an outstanding resistance to proteolytic degeneration.<sup>17</sup> Because natural elastin turnover is exceptionally low,<sup>34</sup> we hypothesize that PGG may remain bound to vascular elastin for extended periods of time after application, sufficient to maintain resistance to enzymes and to deter AAA progression. However, more studies are required to validate this hypothesis.

The mechanisms of aneurysmal dilatation in this animal model are not understood, but we hypothesize that progressive elastin degradation leads to loss of elastic recoil under normal blood pressure, which slowly leads to an increase in

measured external diameter. This arterial expansion was accompanied by increased thickness of the media layer in the saline controls. Because these parameters were significantly reduced in PGG-treated aortas, we are tempted to speculate that direct interaction of PGG with elastin fibers preserves their integrity and helps to maintain mechanical properties of aneurysmal aortas. More detailed studies are categorically needed to better understand the mechanisms of AAA and the interaction of PGG with components of diseased aortas.

Aneurysmal aorta consists of both intact and degraded elastin fibers. For potential clinical applications, we provide supportive evidence that PGG is capable of preventing degeneration of intact elastin fibers. This may account for the prevention of AAA formation in experiment 1 and the lack of AAA progression in experiment 2. However, it is not known whether PGG can interact with partially degraded elastin fibers and stabilize them against further degeneration.

Advanced stages of AAA also involve collagen degeneration, which in turn may contribute to fatal ruptures in terminal stages of AAA.<sup>35</sup> Although polyphenolic tannin binding to collagen has been demonstrated before,<sup>36</sup> it is not known at this point whether PGG also binds to aortic collagen *in vivo* and may possibly influence the outcome of developing AAA, especially in regard to preventing late AAA ruptures. These issues are currently under investigation in our laboratory. In our studies, an acute application of PGG to rat aortas did not directly interfere with model-related pathogenesis; thus, PGG-mediated inhibition of AAA development and progression possibly is due to direct stabilization of elastin rather than to inhibition of enzyme activities.

### Conclusions

Maintaining the integrity of the aortic wall is vital for the prevention of AAA development. Acute localized periadventitial delivery of noncytotoxic concentrations of PGG inhibits elastin degeneration, attenuates aneurysmal diameter expansion, and hinders development of AAA in an established animal model. PGG binds strongly and specifically to arterial elastin, and in doing so, it preserves elastic lamina integrity and architecture despite the presence of high levels of MMPs derived from inflammatory cells. Approaches that target stabilization of the aortic extracellular matrix in aneurysm-prone arterial segments hold great potential toward development of safe and effective therapies for AAAs.

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### Disclosures

None.

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### CLINICAL PERSPECTIVE

Aortic aneurysms (AAs) are degenerative diseases characterized by destruction of arterial architecture and subsequent dilatation that may eventually lead to fatal ruptures. Aneurysms grow over a period of years and pose great health risks as a result of the potential to rupture, which can be fatal in >80% of cases. AAs are a serious health concern for the aging population, among the top 10 causes of death for patients >50 years of age. Currently, the sole treatment of AA is surgical replacement of the diseased artery or endovascular stent graft repair. However, reported postoperative survival rates can drop to only 50% at 10 years. Nonsurgical treatment of aneurysms does not currently exist; thus, a dire need exists for an evidence-based approach to stop this pathological process. The onset and progression of AAs are associated with elastin degradation by proteinases, which, in turn, are derived from activated vascular cells and infiltrating inflammatory cells. Using an aortic injury model that mimics human AA pathology, we show here that periarterial delivery of noncytotoxic concentrations of pentagalloyl glucose, a pharmacological agent capable of stabilizing elastin, significantly reduces AA development and controls diameter expansion by limitation of tissue degeneration. Pentagalloyl glucose delivery provides prospects for the development of clinically applicable therapies as an adjunct or stand-alone procedure that could temper development of a life-threatening pathology and thus affect thousands of patients.